AD_____

GRANT NO: DAMD17-94-J-4342

TITLE: Role of a Novel Antioncogene that Prevents Metastatic Spread of Disease

PRINCIPAL INVESTIGATOR: Timothy E. Kute, Ph.D.

CONTRACTING ORGANIZATION: Bowman Gray School of Medicine
Winston-Salem, North Carolina 27157

REPORT DATE: July 26, 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19960124 025

DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	7/26/95	Annual 1 Jul 94 - 30 Jun 95

4. TITLE AND SUBTITLE
Role of a Novel Antioncogene that Prevents Metastatic Spread of Disease

5. FUNDING NUMBERS
DAMD17-94-J-4342

6. AUTHOR(S)
Timothy Kute , Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Bowman Gray School of Medicine
Winston-Salem, North Carolina 27157

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The aim of this project is to determine the role of a novel antioncogene (nm23) in the spread of cancer. We propose that nm23 prevents metastatic spread of disease by down regulation of proteolytic activity that is necessary for tumor cells to migrate to other parts of the body. We have developed two methods for measurement of nm23 in tumor cells and they are comparable. We have set up test for the cathepsin D and the other components of the urokinase plasminogen activator system. We have measured the expression of these components in 3 cell lines, 8 primary cultures grown in nude mice and more than 50 human primary breast tumors. The relationships of these proteolytic components and nm23 expression are in progress.

We have prepared a new selection vector containing the nm23 gene and have transfected one cell line. Nm23 expression in these transfected cells ranges from 3 to over 50-fold when compared to the non-transfected cells. These cells are presently being analyzed to define affects of over expression of nm23 and relationship to perturbations of proteolytic factors.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Nm23, Proteases, Metastatic Spread of Disease, Breast Cancer	33
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

TK In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

TK For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

TK In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

TK In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

TK In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Timothy Kute 7/26/95

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

The primary cause of death in breast cancer patients is the metastatic spread of disease to vital areas of the body. Many years of research have been spent trying to determine a marker for metastatic potential and the recent work of Dr. Pat Steeg has shed new light on this subject (1). Steeg *et al.* discovered a specific protein called NM23 that has been inversely associated with the metastatic spread of disease. High levels of this protein in breast tissue have been related to a lower probability of disease recurrence and longer patient survival. The transfection of both melanoma and breast cancer cell lines with the NM23 gene was associated with a reduced metastatic potential in nude mice, further supporting the role of NM-23 as an anti-metastatic protein (2,3). The details of these studies have been extensively discussed in the original grant proposal.

The focus of this grant is to evaluate a possible mechanism of NM23's anti-metastatic effect in breast cancer. Our hypothesis is that high levels of NM23 in breast cancer result in the down regulation of proteases. Because cancer invasion and metastasis require the proteolytic degradation of extracellular matrix components, it seems reasonable to propose that the anti-metastatic effect of NM23 is to reduce proteolytic activity. If NM23 does regulate protease expression, it would be of interest to know which proteases are affected, since this information might facilitate future cancer treatments to prevent metastatic disease.

There are two forms of the NM23 protein called H1 and H2 and they have high sequence homology to the alpha and beta forms respectively of a specific enzyme called nucleoside diphosphate kinase (NDP kinase) (4,5). This enzyme has been found in cells from many different sources including the fruit fly larvae, plants and human cells. MacDonald *et al.* demonstrated that NM23-H1 is phosphorylated on two sites: a histadine 122 which is the catalytic site for the enzymatic activity and a serine 44 which can be regulated by cyclic AMP (6). They have shown that the catalytic activity is not related to metastatic spread of disease while the serine autophosphoralation is related to the metastatic spread of disease. This latter phosphorylation suggests that the mechanism of action of NM23-H1 might be related to signal transduction where kinase activity is very important.

Further studies have indicated that cells transfected with NM23-H1 gene show an inhibition of cell motility (7), reduced growth rate and an increase in the production of basement

membrane components (8). In a recent international meeting on NM23/NDPK, Dr. Steeg demonstrated that mutations in the NM23 resulted in increased cell mobility. This is interesting since there are now reports that mutations in NM23 do exists in cancer cells (9).

Other workers have demonstrated that NM23-H2 is >90% homologous to a c-myc transcriptional regulator (10). Postel *et al* suggest that NM23-H2 may regulate the metastatic spread of disease by acting as a transcriptional regulator of genes associated with metastatic disease (10). Other data from transfection experiments (3) and the use of specific antibodies to H2 (11) would suggest that the H2 form is not related to metastatic spread of disease. In conclusion, the current papers in the literature would indicate that the mechanism of action of NM23 on metastatic spread of cancer cells is unknown.

The goal of the present work is to investigate the hypothesis that NM23 protein can down regulate the proteolytic system as a mechanism of preventing the metastatic spread of breast cancer. In this investigation, we are performing both *in vivo* and *in vitro* studies to investigate this hypothesis. In the *in vitro* studies, we are looking at the correlation of NM23-H1 protein expression with specific proteolytic proteins such as cathepsin D, urokinase plasminogen activator (UPA), UPA inhibitor (PAI-1), and the UPA receptor. Each of these factors has been related to cancer metastasis as well as shorter disease free and overall survival (12-16).

The *in vivo* studies will involve the characterization of the metastatic potential of the NM23-H1 transfected cell line MDA-MB-231 compared to the non-transfected cell line in nude mice. These transfected cells will also be used to evaluate how changes in NM23-H1 protein expression are related to changes in proteolytic enzyme expression in tissue culture conditions.

METHODS

A: MEASUREMENT OF NM23

The key component of this grant is the measurement of NM23-H1 protein expression. The original research proposal was to evaluate the levels of NM23-H1 using an ELISA protocol with the help of a commercial company (Oncologex). Three antibodies were available at the time of this proposal; two polyclonal antibodies that recognized NM23-H1 and H2 forms of the protein, and one monoclonal antibody that recognized only the NM23-H1 protein. Preliminary evidence suggested that the ELISA procedure could be adapted to measure NM23-H1 protein levels in breast tumor cytosol extracts. The specificity of the procedure relied on using the

monoclonal antibody for NM23-H1 as the "catching antibody.". The sandwich ELISA procedure subsequently used one of the polyclonal antibodies (of a different species specificity) to "sandwich" the NM23-H1 protein to the ELISA plate. Detection was finally evaluated using an anti-rabbit biotinylated antibody.

The preliminary results that were published with the research proposal were obtained using a polyclonal rabbit anti-NM23-H1 antibody whose production was discontinued. Oncologix provided us with a large quantity of another polyclonal antibody that was of a much higher titer than the original antibody. The ELISA procedure using the new antibody was never perfected. The ongoing problem was a very high non-specific binding that could not be eliminated without the loss of the specific signal. Given the complexity of the ELISA sandwich procedure, it took a series of steps to eliminate the possible cross-reacting species responsible for the non-specific binding. Several factors were eliminated sequentially, including anti-Rh factors present in the polyclonal anti-NM23-H1 antibody against either mouse, rabbit or goat. The non-specific binding could not be eliminated with large excesses of either rabbit, goat or mouse sera. The conclusion was that the polyclonal antibody was reacting in some epitope specific or non-specific manner with the monoclonal "catching antibody" and therefore could not be removed without loosing the specific signal. We were left with the option of trying to obtain another polyclonal anti-NM23-H1 antibody, removing the cross-reactivity by further purification and cross-adsorption of the polyclonal antibody, or using an alternate method to measure NM23-H1.

The decision to use western blot analyses to quantitatively measure NM23-H1 was made based on immune-precipitation data using the monoclonal antibody. The immune-precipitation of tumor extracts using the monoclonal antibody to the NM23-H1 protein, showed some NM23-H2 cross-reactivity. Since NM23-H2 has not been shown to correlate with metastatic breast cancer and should be measured in addition to NM23-H1, we chose to measure NM23-H1 levels using the western blot procedure that will be fully described below.

The advantages of the western procedure over the ELISA procedure in this case include: 1. Increased sensitivity. NM23-H1 levels as low as 0.04 ng can be detected by western blot analyses using the enhanced chemiluminescence procedure and biotin-streptavadin HRP. The best sensitivity obtained by the ELISA procedure was 1.56 ng of NM23-H1. 2. The western protocol has the additional ability to measure both NM23-H1 and H2 isoforms since

these are adequately separated on electrophoresis to allow independent quantification. The only disadvantage is that the western procedure is somewhat more labor-intensive, however, the advantages seem to warrant the additional effort in that they provide more accurate and sensitive analysis of NM23-H1.

A-1 USE OF WESTERN PROCEDURE

The procedure for the western blot analysis of NM23-H1 is described briefly. Patient tumor cytosol extracts (30 -50 µg/lane) are boiled in SDS-lysis buffer and loaded onto a 15% SDS-PAGE discontinuous system. Electrical current is applied to facilitate the separation of proteins according to their molecular weight. Each gel contains purified NM23-H1 protein standards ranging from 2 ng-100 ng as well as molecular weight markers. After sample separation, the proteins are transferred to a nitrocellulose membrane using electrical current. Membranes are then blocked using a 5% casein-Tris buffer and the blots are incubated overnight at 4°C with 50 µg polyclonal anti NM23 antibody. The blots are then washed and the primary antibody is labeled with a biotinylated anti-rabbit antibody. Further washing is followed by the labeling of the biotinylated antibody with streptavadin horseradish peroxidase enzyme. Finally, the blots are emersed in a chemiluminescent reagent and the NM23 protein labeled with HRP is detected on x-ray film.

Quantification of the levels of NM23-H1 in patient tumor extracts is performed using the standard curve of the known quantities of NM23-H1 and scanning laser densitometry. Known quantities of NM23-H1 are plotted against optical density x mm and a standard curve is plotted for each experiment. Examples of typical standard curves and the accompanying westerns are shown in Figures (1 and 11).

A-2 IMMUNOHISTOCHEMISTRY FOR NM23

The immunohistochemical analyses of NM23-H1 in breast cancer patients are performed according to standard procedures. Briefly, human breast tumors are fixed in 10% buffered formalin and embedded in paraffin according to standard procedures. Paraffin sections (4-5 micron) are cut from each breast tumor specimen, deparaffinized in xylene and rehydrated in a graded series of ethyl alcohol. The tissues are then washed in distilled water for 2-5 minutes.

The monoclonal antibody NM-301 (Oncogene Science) recognizes NM23 H1 in human breast tumor specimens. The optimum

antibody concentration was established at 5 µg/ml. Tissue specimens are analyzed for NM23-H1 using an automated staining system (Ventana) and manufacturer recommended reagents necessary for the detection of primary antibody using AEC (3-amino ethylcarbazole) as the chromogen. Pretreatment of the tissues with alkaline protease (protease 2 supplied by Ventana) for 8 minutes has been shown to enhance the sensitivity of the detection of NM23-H1.

Human breast carcinoma is the recommended positive control and skin the negative. Additionally a serum control is included as a negative control for each patient specimen

A pathologist at the Bowman Gray School of Medicine, (Dr. K. Geisinger) is performing the analyses of the immunohistochemistry for each specimen. The criteria for the analysis of each specimen include; the staining intensity of the breast tumor (0-3+), the percentage of tumor that is staining at the given intensity and the percentage of tumor in the specimen.

B: MEASUREMENTS OF PROTEOLYTIC COMPONENTS

B-1 ELISA PROCEDURES FOR THESE COMPONENTS

Human breast tumor extracts are being analyzed for a number of proteolytic factors including UPA, UPA-receptor, PAI-1 and Cathepsin D. The cathepsin D analyses will be performed at the Bowman Gray Medical School while the other markers will be analyzed at the Finsen Institute (Copenhagen, Denmark). We have a collaboration with this group because they already have published in this area and the ELISA procedures for these markers are routinely done in their laboratories (13).

Cathepsin D is being analyzed by an immunoradiometric assay (CIS biointernational). This assay is a solid phase two site immunoradiometric assay using sterically remote antigenic sites on the cathepsin D molecule. The immunoassay quantifies total cathepsin D, including the 52 kD, 48 kD and 34 kD forms of this protease in breast tumor lysates. One monoclonal antibody is bound in solid phase on test tubes, the other antibody is radiolabeled using ^{125}I as the tracer. We have published clinical results using this method so these data will not be discussed in this report (16).

B-2 IMMUNOHISTOCHEMISTRY PROCEDURES

Cathepsin D is also being analyzed by immunohistochemical techniques. The cathepsin D monoclonal antibody is produced by Triton diagnostics. Immunohistochemical analyses are performed according to usual techniques using the Ventana and AEC detection as described above. The monoclonal antibody to cathepsin D recognizes both the 34 kD and the 48 kD forms of cathepsin D. The cathepsin D antibody is diluted to 0.125 µg/ml for immunohistochemical analyses.

We are currently working on the immunohistochemical techniques for UPA, PAI-1 and UPA receptor. We have the antibodies and have the slides ready for analysis. Preliminary data would suggest that PAI-1 expression can be measured using a monoclonal antibody from American Diagnostica in paraffin embedded blocks.

C: TRANSFECTION EXPERIMENTS/ANIMAL STUDIES

C-1 PREPARATION OF PROBE

As discussed in the grant proposal, we plan to use two breast cancer cell lines that are metastatic and contain a marker gene (i.e. LacZ). Since these cells were already neomycin selected and the vector that was obtained from Dr. Pat Steeg contained the neomycin resistant gene, it was necessary to make a new probe that had a different selection gene. This was performed by removing the NM23-H1 gene and inserting it into a hygromycin resistant vector. The NM23-H1 hygromycin vector has been synthesized, and analyzed for orientation using restriction enzyme digestion and agarose gel electrophoresis. The details of the procedure are in the grant proposal.

C-2 TRANSFECTION OF MDA-MB-231 CONTAINING THE LACZ GENE

The transfection of the Lac Z containing cells with NM23 was performed by the standard calcium phosphate precipitation technique. Briefly, 20-30 ug of purified NM23-H1 vector was precipitated onto 500,000 log phase MDA-MB-231 cells grown on 100 cm^2 tissue culture dishes. The cells were incubated with the vector DNA for 12-18 hours and then the excess vector was removed by gentle washes with complete medium. The cells were then

allowed to grow for 24 hours before the selection medium was applied. The LD99.9 (0.66 mg/ml) for hygromycin was evaluated using colony forming assays, and this dose was applied continuously to the dishes until transfected clones appeared.

C-3 ATHYMIC NUDE MOUSE MODEL

We are using the athymic nude mouse model for the measurement of metastatic breast cancer as described by others (17,18). We have used two sources of nude mice: 1. Our in-house nude mouse breeding facility and 2. NCr nude mice, purchased from the NIH. The athymic nude mice are given a subcutaneous injection of 10^6 cells in the lower mammary pads. The animals are then followed for 2 to 3 months and the measurement of primary tumor growth is obtained. Any animals that appear sick or have tumors that become necrotic are sacrificed. The primary and any metastatic tumors are removed for histological analyses at the time of the mouse sacrifice. We have also performed the X-gal labeling studies for the tumor marker gene (i.e. LacZ) (18). We have completed a major study using 10 animals in two different groups. The first group of animals was injected with the MDA-MB-231 while the second group of animals was injected with the MDA-MB-435 cell line.

RESULTS

NM23 EXPRESSION IN DIFFERENT CELL TYPES

We have evaluated NM23-H1 protein levels in primary breast cancer tissues, breast cancer cell lines, and nude mouse xenografts of primary human tumors using western blot analyses (17). Table 1 gives a description of the number of samples done and the results obtained. The levels of the three cell lines are very different. MCF-7 cells express the most and are the least metastatic of the three cell lines. MDA-MB-231 and MDA-MB-435 have much lower levels of NM23-H1 and are reported to be metastatic in the athymic nude mouse model. There is a broad range of values for NM23-H1 in the different solid tumors derived from patients and from primary human tumors grown in nude mice. Figure 1 demonstrates our analysis of NM23 expression in breast cancer tissues. The first 5 lanes are our standard curve while the remaining lanes represent extracts from different breast tumors. The upper band co-migrating

with our standard represents NM23-H1 while the lower migrating band represnts NM23-H2.

Since NM23-H1 is being measured by two different procedures, a comparison of the two different methods is shown in Figure 2. A good correlation was seen between the results obtained by immunohistochemical analysis and the values obtained by western analysis. Given the limited sample size, (n=39) the correlation between the immunohistochemical and western analyses suggest that both methods are comparable for the measurement of the NM23-H1 protein.

NM23-H2 levels were measured concurrently with NM23-H1 protein levels. The correlation of NM23-H1 with NM23-H2 is shown in Figure 3. Since NM23-H1 and H2 proteins share considerable sequence homology and are coordinately regulated, it is not surprising that there is a good correlation between the levels of these proteins in breast tumor extracts.

Our experience in the analyses of breast cancer patient specimens, tissue culture cell lines and cell lysates from primary human breast cancer grown in nude mice, has shown that in some cases one the these methods may be superior to the other. In the case of human tumor cell lines grown in nude mice models, the western blot technique has been shown to provide superior information since there is a high nonspecific tissue reactivity of mouse-derived tumors and the monoclonal antibodies. These tissues are easily analyzed for NM23-H1 protein using western blot techniques. Immunohistochemistry may be more valuable in the analyses of human breast carcinoma, particularly in patients with very small tumor size where extracts may not be available. Some representative examples of immunohistochemical staining for breast cancer patient's tumors are given in Figure 4. Our analysis would indicate that most of the staining is cytoplasmic and is found in both invasive and non-invasive tumor cells. The panels represent different intensities of staining and it should be noted that the negative controls have no staining and therefore were not presented in this figure.

COMPARISON OF CATHEPSIN D WITH NM23 EXPRESSION

A total of 86 patient samples have been analyzed for the expression of cathepsin D using the immunoradiometric assay. A preliminary analysis of the correlation between the ELISA cathepsin D levels and the values for NM23-H1 protein by western blot

analysis is presented in Figure 5. These preliminary data suggest that total cathepsin D expression is not related to NM23-H1 expression. Similar results were obtained when cathepsin D was analyzed by immunohistochemistry and correlated to immunohistochemical or western blot-derived values of NM23-H1 (data not shown).

Breast tumors have variable staining for cathepsin D using immunohistochemical techniques. Additionally, certain tumors have been shown to have intense staining in the stromal cells within the tumor. These observations are being documented to facilitate data interpretation at the end of the study. The intense staining of macrophages and stromal cells in solid tumors as well as the recognition of the 52 kD pro-Cathepsin D by ELISA may account for the lack of correlation between the two different assays for cathepsin D (see Figure 6) and might be the reason why this specific protease does not correlate with NM23-H1 expression.

COMPARISON OF THE UPA SYSTEM WITH NM23 EXPRESSION

Our preliminary results on the relationship of NM23-H1 expression by Western blot analysis and the expression of UPA. PAI-1 and UPA receptor from the same tumor extract is presented in Figure 7. There is no correlation between the NM23-H1 protein and any of these factors in this preliminary data. We have just sent an additional 64 samples to Denmark for further analysis.

Analyses of the UPA system in the breast cancer specimens are also being performed by immunohistochemical analyses. This work is in progress and titrations have been performed to determine the optimization conditions for these analyses.

RELATIONSHIP OF NM23 TO METASTATIC SPREAD OF DISEASE

One of the specific aims of this study is to evaluate the relationship between the expression of the NM23-H1 protein and the metastatic potential of breast cancer. Toward this goal we have been collaborating with Dr. R. Mehta to measure the levels of NM23-H1 in primary human breast tumor cell lines that have known metastatic potential in nude mice (17). We have analyzed eight solid tumor xenografts for NM23-H1 and cathepsin D expression using western blot analyses and immunoradiometric assay procedures, respectively. The relationship between the expression of NM23-H1 and the metastatic potential of these primary cell line-derived tumors is

shown in Figure 8. Assays on these samples were done multiple times in order to obtain standard error values. Two of the three xenografts with the highest NM23-H1 are not metastatic (A and B) while 4 of the metastatic tumors have lower levels of NM23-H1 (D, E, F and H). One tumor that is considered occasionally metastatic has high levels of NM23-H1 (G) and one metastatic tumor (C) has high levels of NM23-H1 protein. Statistical analyses of these xenografts show that metastatic tumors C and D are not different from the non-metastatic tumors B or A. There does appear to be a statistically significant difference between the non-metastatic (A and B) and occasionally metastatic (G) tumors versus metastatic tumors E, F and H.

The relationship between cathepsin D concentration and metastatic potential is shown in Figure 9. From this limited data set where only single assays were performed, there does not appear to be a relationship between the metastatic spread of disease and cathepsin D levels .

TRANSFECTION OF THE NM23 GENE INTO MDA-MB-231.

The purpose of the transfection study is to evaluate the metastatic potential of human breast tumor cell lines expressing elevated levels of NM23-H1 protein in a nude mouse tumor model. The parental MDA-MB-231 breast tumor cell line contains very low levels of NM23-H1 (table 1) and has been shown by others to have metastatic potential in nude mice (18). Double transfection of these cells with the LacZ and NM23-H1 genes will provide a means to determine whether increasing the level of NM23 will reduce the metastatic potential of these cells in nude mice. The expression of the Lac Z gene will facilitate the detection of micrometastatic lesions in the nude mouse by acting as a reporter gene. It is, however, critical to prove that the parent cell line is metastatic.

In a preliminary experiment, we have shown micrometastatic disease in 30% of nude mice injected with MDA-MB-231 parental cell line. Further experiments are in progress to establish a good baseline for the metastatic potential of the parental cell line. We have similarly shown a 50% metastatic potential for the parental breast cell line MDA-MB-435. In our preliminary experiments we were trying to evaluate metastatic disease using gross inspection. We have subsequently determined that both cell lines MDA-MB-231 and MDA-MB-435 form micrometastatic lesions in lung sections that can be visualized by analyzing serial sections of lung tissue. Figure 10

demonstrates what micrometastic disease looks like in these lung tissues after staining with a specific substrate for LacZ (i.e. X-gal).

A more direct manner of evaluating the effect of NM23-H1 protein levels on the expression of proteolytic factors is also in progress. The MDA-MB-231 cell line was transfected with NM23-H1 and Lac Z genes. A total of 47 clones were obtained and from these 12 clones were selected that expressed low, moderate and high levels of NM23-H1 as determined by western blot analysis. A representative western blot for NM23-H1 expression of the transfected clones is shown in Figure 11.

The level of expression of NM23-H1 and cathepsin D in the transfected clones is shown in Figure 12. It would seem that cathepsin D is higher in the parent cell line compared to any of the clones but that the relationship of increase expression of NM23-H1 to cathepsin D is not evident in these studies. This agrees with what has been demonstrated using primary human breast cancer tissues as described above.

CONCLUSIONS

NM23-H1 NM23-H2 cathepsin D, UPA, PAI-1 and UPA-R proteins have been analyzed in clinical tumor extracts, breast cancer cell lines, transfected cells and nude mouse-derived tumors using western blot, ELISA's and immunohistochemical analyses. Our data demonstrate a relationship between NM23-H1 values obtained by Western analysis and those done by immunohistochemistry (Figure 2). We also demonstrated a direct correlation between the levels of NM23-H1 and NM23-H2 ((Figure 3).

We have also shown that cathepsin D levels measured by either method are not related to NM23-H1 levels measured by western blot (Figure 5) or immunocytochemistry (data not shown). We have also demonstrated that NM23-H1 expression does not seem to be related to cathepsin D in cells lines that have been tranfected and expressed different amounts of NM23-H1 (Figure 12).

The ELISA data for UPA, UPA receptor and PAI-1 was performed on 55 breast tumor extracts and correlations performed to compare these proteases and protease inhibitors to NM23-H1 protein. The preliminary results of these data suggest that NM23-H1 is not related to the expression of the UPA system components (Figure 7).

We have also compared NM23-H1 expression to metastatic potential using a series of human breast tumor xenografts. Our

results suggest that in most cases high expression of NM23-H1 is found in non-metastatic tumors while low expression is found in metastatic tumors (figure 8). Preliminary data for these 8 xenographs would suggest no relationship of cathepsin D to metastatic spread of disease (figure 9).

We have modified the vectors containing the NM23-H1 gene and transfected the MDA-MB-231 cell line. From these transfection studies, we have selected 12 clones that have between 3 and 55 fold overexpression of NM23-H1 protein (Figure 11). We are presently working with Goodwin Institute for Cancer Research to transfect another highly metastatic cell line (19). If this study is successful, we will have another distinct breast cancer cell line to evaluate in the analyses of the metastatic potential of breast cancer and the levels of NM23-H1. We have decided not to transfect MDA-MB-435 since these cells have already been transfected and this work has been published (3). We have now perfected our athymic nude mouse model and can detect micrometastatic disease in the lungs (Figure 10) and are presently working on the growth and metastatic spread of disease for parent and NM23 transfected MDA-MB-231 cell lines in this model.

Year two goals are to continue to collect data for NM23-H1 and the proteolytic factors (UPA, UPA-R, PAI-1 and cathepsin D) by both the quantitative and semi-qualitative procedures. Additional goals include the characterization of the nude mouse metastatic model and analyses of the NM23-H1 transfected clones' metastatic potential.

REFERENCES

1. Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, Sobel ME. Evidence for a Novel Gene Associated With Low Tumor Metastatic Potential. J Natl Cancer Inst 1988;80:200-204.

2. Leone A, Flatow U, King CR, Sandeen MA, Margulies MK, Liotta LA, Steeg PS. Reduced tumor Incidence, Metastatic Potential, and Cytokine Responsiveness of *NM23-H1*-Transfected Melanoma Cells. Cell 1991;65:25-35.

3. Leone A, Flatow U, VanHoutte K, Steeg PS. Transfection of human *NM23-H1*-H1 into the human MDA-MB-435 breast carcinoma cell line: Effects on tumor metastatic potential, colonization and enzymatic activity. Oncogene 1993;8:2325-2333.

4. De La Rosa A, Williams RL, Steeg PS. Nm23/nucleoside diphosphate kinases: Toward a structural and biochemical understanding of its biological functions. Bioassays 1995;17:53-62

5. Stahl JA, Leone A, Rosengard AM, Porter L, King CR, Steeg PS. Identification of a Second Human *NM23-H1* Gene, *NM23-H1*-H2. Cancer Research 1991;51:445-449.

6. Mac Donald NJ, De La Rosa A, Benedict MA, Perez-Freije JM, Krutsch H, Steeg PS. A serine phosphorylation of NM23, and not its nucleoside diphosphate kinase activity, correlates with suppression of tumor metastatic potential. J Biol Chem 1993;268:25780-25789

7. Kantor JD, McCormick B, Steeg PS, Zetter BR. Inhibition of Cell Motility after *NM23-H1* Transfection of Human and Murine Tumor Cells. Cancer Research 1993;53:1971-1973.

8. Howlett AR, Petersen OW, Steeg PS Bissell MJ. A novel function for the NM23-H1 gene: overexpression in human breast carcinoma cells leads to the formation of basement membrane and growth arrest. J of Natl Cancer Inst 1994;86:1838-1844

9. Wang L, Umesh P, Ghosh L, Chen HC and Banerjee S. Mutation in the NM23 gene is associated with metastasis in colorectal cancer. Cancer Res 1993;53:717-720

10. Postel EH, Berberich SJ, Flint SJ, Ferrone CA. Human c-*myc* Transcription Factor PuF Identified as *NM23-H1*-H2 Nucleoside Diphosphate Kinase, a Candidate Suppressor of Tumor Metastasis. Science 1993;261:478-480.

11. Tokunaga Y, Urano T, Furukawa K, Kondo, H, Kanematsu T, Shiku H. Reduced expression of NM23-H1-H1 but not of NM23-H1-H2, is concordant with the frequency of lymph-node metastasis of human breast cancer. Int. J. Cancer: 55, 66-71, 1993.

12. Schmitt M, Janicke F, Graeff H. Tumor-associated proteases. Fibrinol 1992;6:3-26

13. Grøndahl-Hansen J, Christensen J, Rosenquist C, Brünner N, Mouridsen HT, Danø K, Blichert-Toft M. High Levels of Urokinase-type Plasminogen Activator and Its Inhibitor PAI-1 in Systolic Extracts of Breast Carcinomas Are Associated with Poor Prognosis. Cancer Research 1993;53:2513-2521.

14. Duffy MJ, Reilly D, O'Sullivan C, O'Higgins N, Fennelly JJ, Andreasen P. Urokinase-Plasminogen Activator, a New and Independent Prognostic Marker in Breast Cancer. Cancer Research 1990;50:6827-6829.

15. Rochefort H. Cathepsin D in breast cancer. Breast Cancer Research and Treatment 1990;16:3-13.

16. Kute TE, Shao LM, Sugg NK, Long RT, Russell GB, Case LD. Cathepsin D as a Prognostic Indicator for Node-negative Breast Cancer Patients using Both Immunoassays and Enzymatic Assays. Cancer Research 1992;52:5198-5203.

17. Mehta RR, Graves JM, Hart GD, Shillkaitis A, Das Gupta TK. Growth and metastasis of human breast carcinomas with Matrigel in athymic mice. Breast Cancer Research and Treatment 1993;25:65-71

18. Brünner N, Thompson EW, Spang-Thomsen M, Rygaard J, Danø K, Zwiebel JA. LacZ Transduced Human Breast Cancer Xenografts as an *in vivo* Model for the Study of Invasion and Metastasis. Eur J Cancer 1992;28A(12):1989-1995.

19. Hurst J, Maniar N, Tombarkiewicz J, Lucas F Roberson C, Steplewski Z, James W, Perras J. A novel model of a metastatic human breast tumor xenograft line. Br.J. Cancer 68: 274-276, 1993.

FIGURE LEGENDS

FIGURE 1: The expression of NM23-H1 and H2 levels in Breast cancer tumor extracts: Equal protein (50ug per lane) was loaded onto 12.5% acrylamide gels and analyzed by SDS-PAGE. Lanes 1-5 represent known NM23-H1 standards and contain 100,75,50,30 and 20 ng of purified protein, respectlvely. Lanes 6-14 contain NM23 extracted from homogenized patients breast tumors. The upper band represents NM23-H1 and the lower band in NM23-H2. The ng/ug values for NM23 expression were derived from the standard curve shown above.

FIGURE 2: The relationship between the levels of NM23-H1 obtained by western blot and immunohistochemical analyses. NM23-H1 protein levels obtained by western blot analyses were compared to the levels of intensity of NM23-H1 as measured by immunohistochemistry. A total of 39 patients were analyzed by both immunohistochemical and western blot procedures. Patients with less than 0.2 ng/ug of NM23-H1 by western analyses, showed no positivity by immunohistochemistry. The mean NM23-H1 levels for a 1, 2 and 3 intensity by immunohistochemical analyses were 0.32, 0.6 and 1.0 ng/ug, respectively by the western blot procedure.

FIGURE 3: Correlation between NM23-H1 and NM23-H2 protein levels in human tumor extracts. Human breast tumor extracts were analyzed by western blot as described in Methods section A1. For each tumor, NM23-H1 and NM23-H2 protein levels were measured using the standard curve for purified NM23-H1. A total of 117 tumors were analyzed by this procedure and regression analyses performed. The data suggest that there is a good correlation between the levels of the NM23-H1 and NM23-H2 proteins.

FIGURE 4: The expression of NM23 staining in breast cancer tissues. Panels A, B, and C 1+,2+,3+ intensity, respectively for NM23 by immunohistochemistry (see color prints enclosed)

FIGURE 5: Correlation between the levels of NM23-H1 protein and the protease Cathepsin D. NM23-H1 in human breast tumor extracts was measured by western blot as described in Methods section A1. The same tumor extracts were used to measure cathepsin D by the ELISA procedure described in Methods section B1. A total of 87 tumors were analyzed by these procedures and regression analyses performed. The data suggest that there is no correlation between the levels of the NM23-H1 protein and cathepsin D.

FIGURE 6: Relationship of Cathepsin D staining intensity by immunohistochemistry and quantitative analysis of cathepsin D by immunoradiometric assay. The details for the analyses are given in the methods. There was no relationship between these two methods for analysis of cathepsin D

FIGURE 7: Correlation between the levels of NM23-H1 Protein and Urokinase plasminogen Activator (UPA) Components. Analyses of the correlation between the levels of NM23-H1 protein and UPA components were performed on 55 human breast tumor extracts. NM23-H1 was analyzed by western blot as described in Methods section A1 and UPA components were analyzed by ELISA (Methods section B1). Regression analysis suggests that the levels of these 3 proteins are not related to NM23-H1 expression.

FIGURE 8: NM23-H1 protein levels in nude mouse xenografts of human primary breast tumors and the relationship to the metastatic potential in nude mice. Eight primary human breast tumors that were grown as nude mouse xenografts were analyzed by the western blot procedure for NM23-H1 protein levels. The values of NM23-H1 were derived from a minimum of eight different experiments and two separate tumor extraction procedures. The tumor xenografts were classified according to their metastatic potential in nude mice (data from Dr. R. Mehta personal communication). The relationships of these two parameters are discussed in the text.

FIGURE 9: Cathepsin D protein levels in nude mouse xenografts of human primary breast tumors and the relationship to the metastatic potential in nude mice. Eight primary human breast tumors that were grown as nude mouse xenografts were analyzed by the radioimmuno assay procedure for cathepsin D. The cathepsin D values were compared to the metastatic potential of the xenografts in nude mice as discussed in Figure 8. No relationship was seen between the levels of cathepsin D and the metastatic potential of these xenografts.

FIGURE 10: Demonstration of micro-metastatic disease in lungs of athymic nude mice using a marker for tumor cells.
MDA-MB-231 (Panel A) and MDA-MB-435 (Panel B) breast cancer cell lines transfected with LacZ were injected into the lower mammary pads of athymic nude mice and after 8 weeks the animals were sacrificed. The lung tissues were then stained for LacZ activity as described by Brunner et al (18) and then sectioned for analysis. The blue chromogen is related to LacZ expression. Arrows indicate areas of metastatic disease. (See Color prints enclosedA)

FIGURE 11: MDA-MB-231 breast cancer clones transfected with NM23-H1. The MDA-MB-231 cell line containing the LacZ gene was transfected with the NM23-H1 hygromycin vector as described in the text. Western blot analysis was performed as described in Figure 1 where lanes 1-4 represent known NM23-H1 standards and contain 100,50,12.5 and 3.1 ng of purified protein, respectively. Lanes 5-13 represent 9 different transfected clones and lane 14 is the parent cell line. The two distinct bands in the transfected clones represent NM23-H1 (top band) and NM23-H2 (lower band). The ng/ug values for NM23-H1 expression in the clones were derived from the standard curve shown above.

FIGURE 12: The relationship between the fold induction of NM23-H1 protein and cathepsin D in MDA-MB-231 NM23-H1 transfected clones. The human breast cancer cell line MDA-MB-231 was transfected with the NM23-H1 gene as described in Methods section C2. The level of NM23-H1 protein expression is shown in this Figure and ranges from 3-55 fold induction. Cathepsin D was also measured in each of the selected clones and the level of expression of cathepsin D and NM23-H1 was compared in this Figure. There is no relationship between the fold induction of NM23-H1 and the levels of cathepsin D in these transfected clones.

ACKNOWLEDGEMENTS

PAPERS/ABSTRACT REPORT

At the present time, we have not published any papers from the work. We have presented a paper at the **INTERNATIONAL NM23/NDP KINASE WORKSHOP** in Paris on Oct 12-13,1995. We are currently writing a paper on our preliminary data which will be submitted to Cancer Research by December. We also have submitted an abstract to the United States and Canadian Academy of Pathology Meeting in Washington and will submit an abstract for the American Association for Cancer Research.

PERSONS PAID ON GRANT

PERSONS	PERCENT EFFORT	POSITION
DR. T KUTE	30%	PI
DR. R RUSSELL	100%	POST DOC.
DR. K GEISINGER	3.6%	CO-INVESTIGATOR
DR. A TOWNSEND	5%	CO-INVESTIGATOR

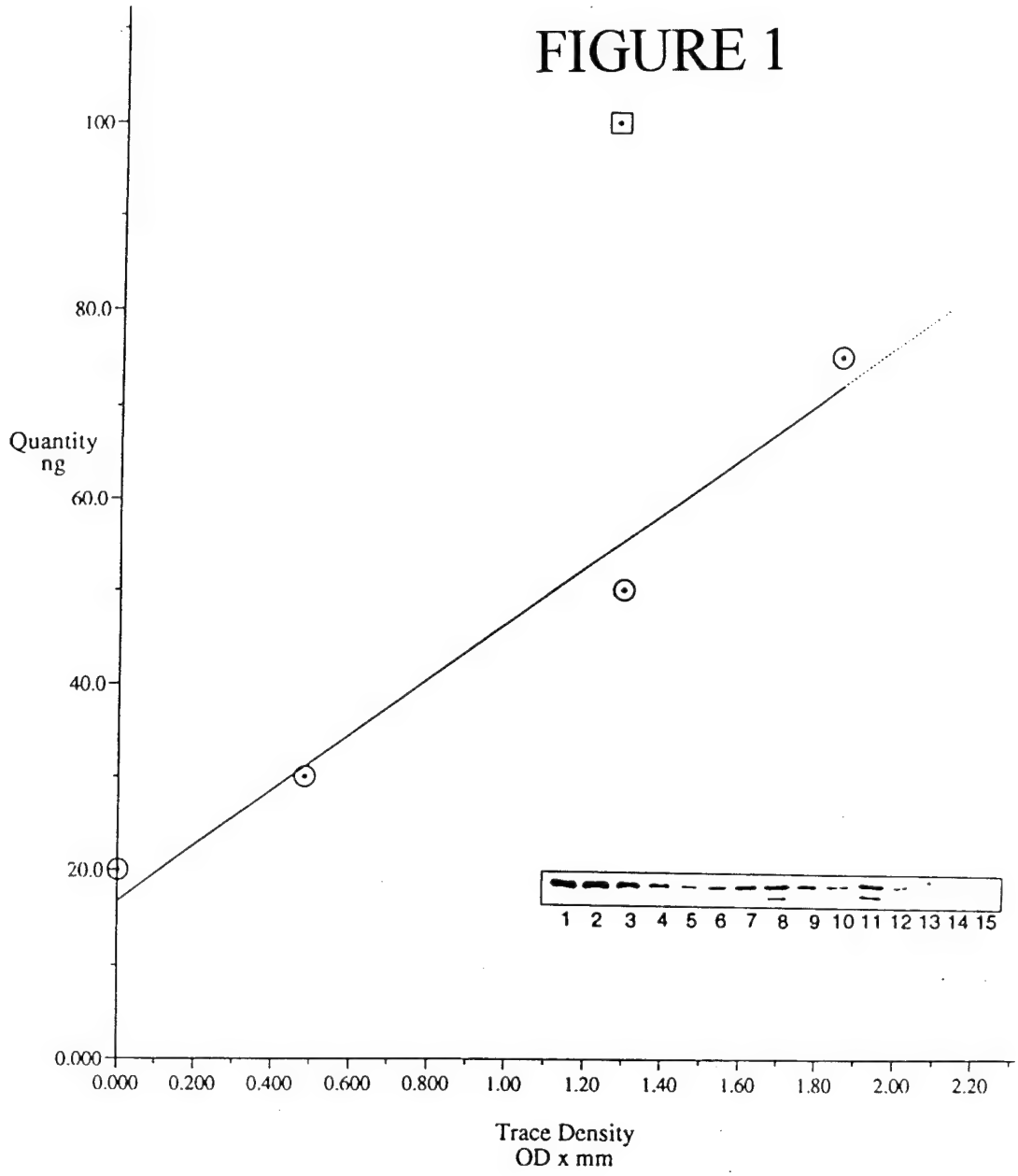
TABLE 1

EXPRESSION OF NM23-H1 IN BREAST CANCER CELL LINES, PATIENT TUMORS AND XENOGRAFTS DERIVED FROM PRIMARY HUMAN TUMORS

CELL TYPE	N	MEAN	RANGE	SE
MCF7	9	2.09	1.27-3.46	0.26
MDA-MB-231	4	0.109	0.014-0.228	0.051
MDA-MB-435	5	0.369	0.085-0.757	0.118
PATIENTS §	119	0.723	0-3.17	0.051
XENOGRAFT ¶	8	0.226	0-0.962	0.029

§ Clinical breast tumor specimens were analyzed by western blot. The numbers represent ng/µg of nm23 in tumor extract.
¶ Human breast cancer specimens were transfered to nude mice. The resulting tumor, grown in nude mice was analyzed for nm23 -H1 by western blot. The numbers represent the ng/µg protein of nm23.

FIGURE 1



RELATIONSHIP BETWEEN NM23 IHC VERSUS WESTERN

FIGURE 2

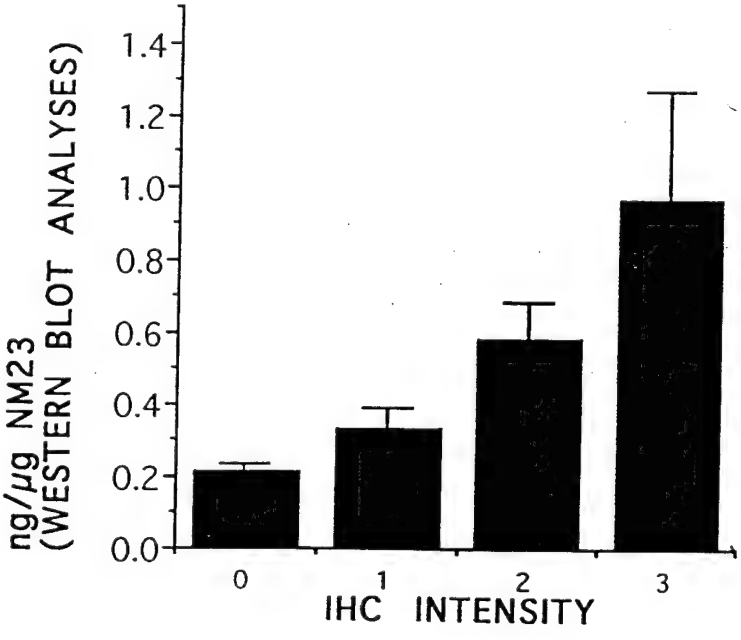


FIGURE 3

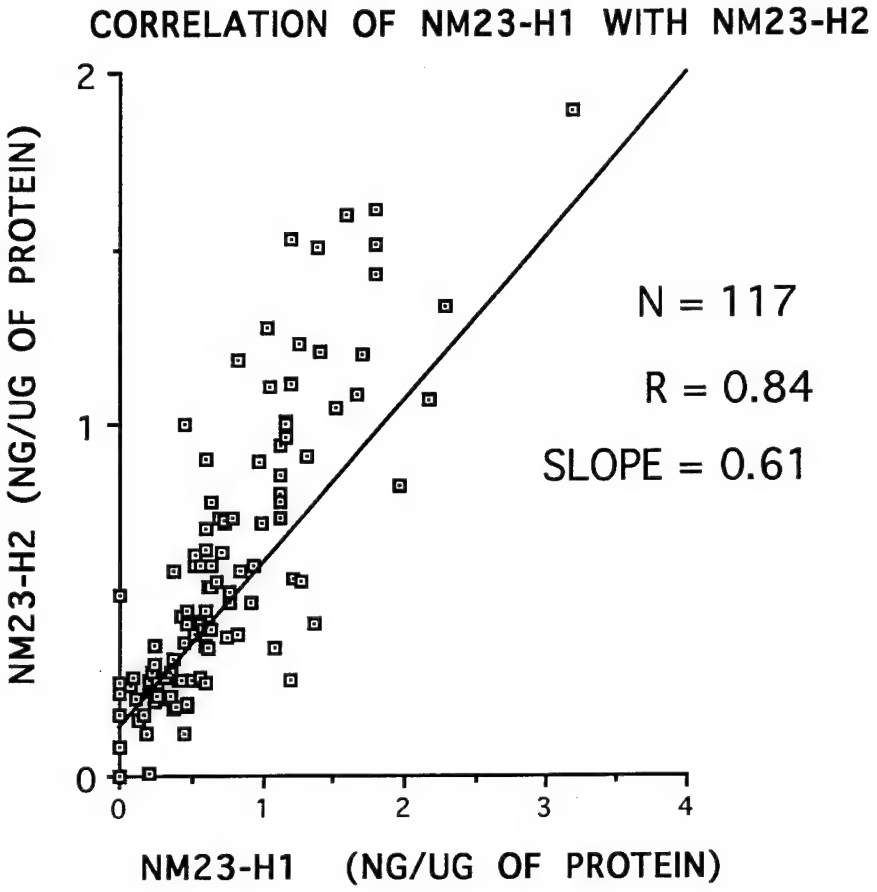

CORRELATION OF NM23-H1 WITH NM23-H2
NM23-H2 (NG/UG OF PROTEIN)
NM23-H1 (NG/UG OF PROTEIN)
N = 117
R = 0.84
SLOPE = 0.61
0
1
2
0
1
2
3
4

Figure 4 A

FIGURE 4 (PANEL A- 1+ INTENSITY)

Figure 4 B

FIGURE 4 (PANEL B- 2+ INTENSITY)

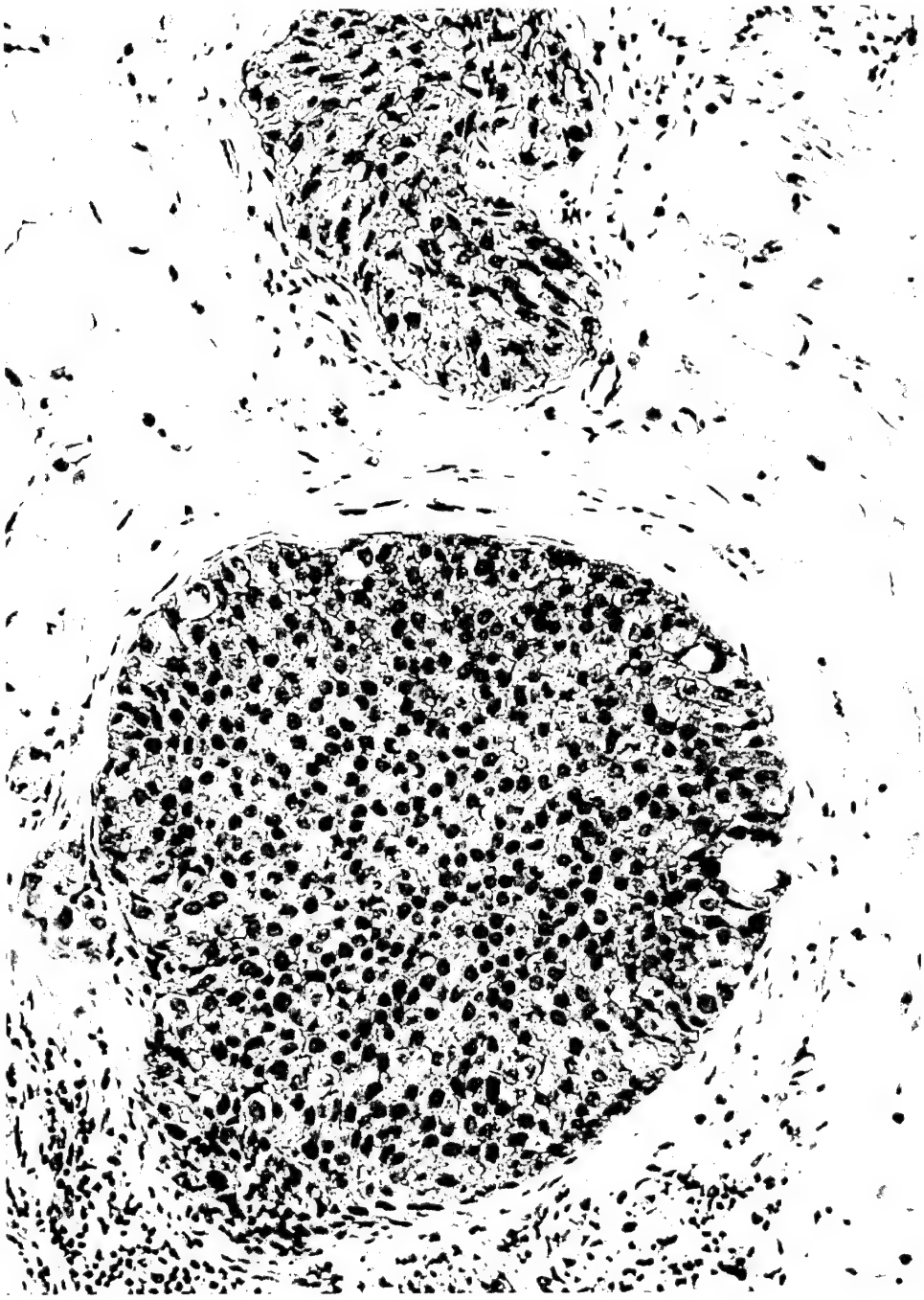


FIGURE 4 (PANEL C- 3+ INTENSITY)

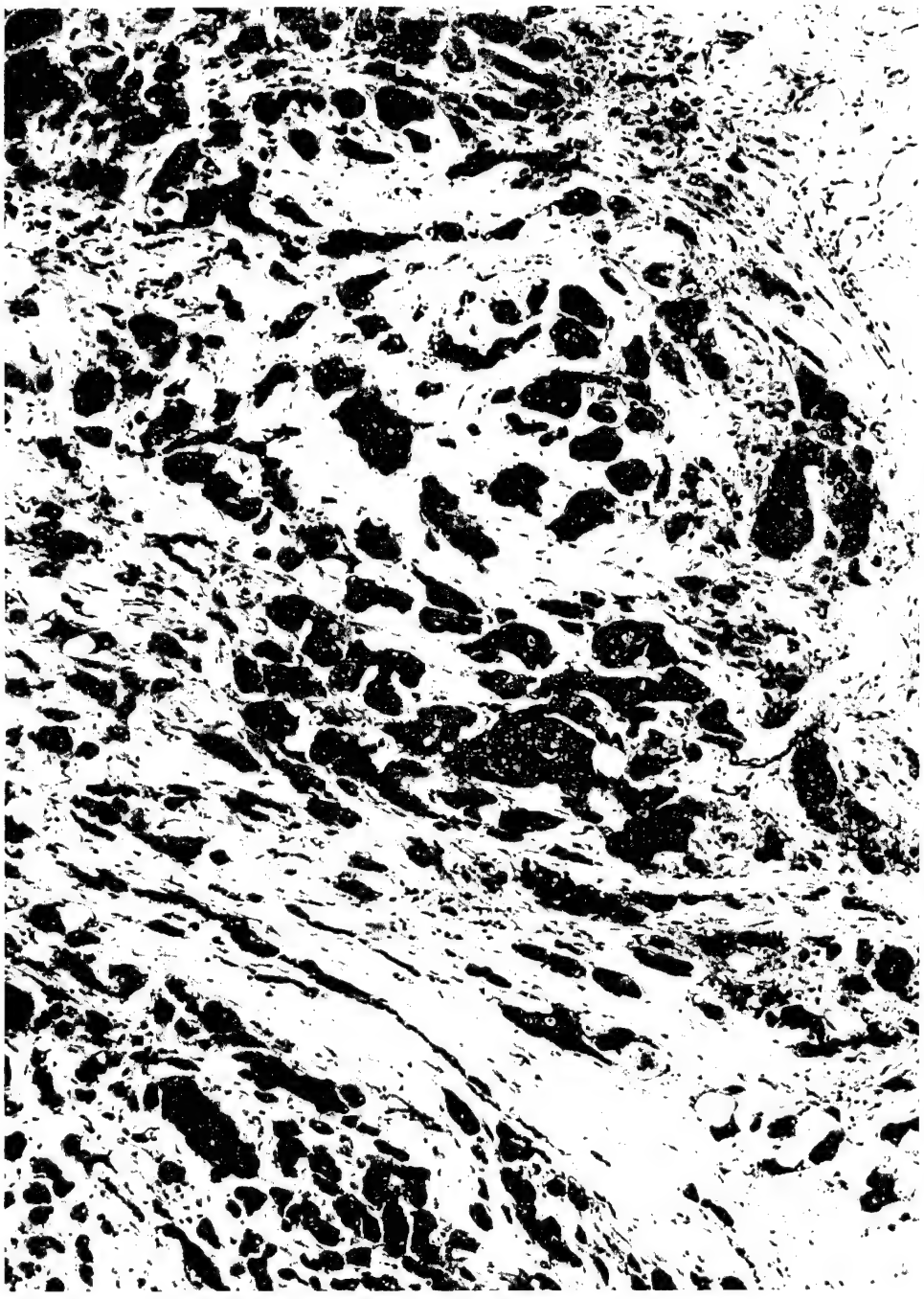


FIGURE 5

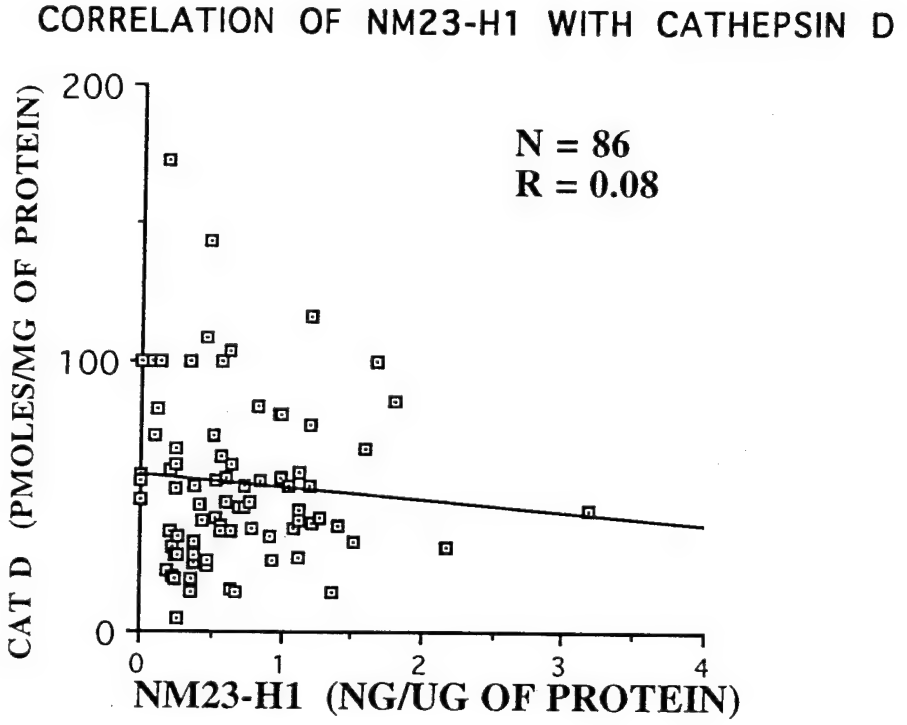


FIGURE 6

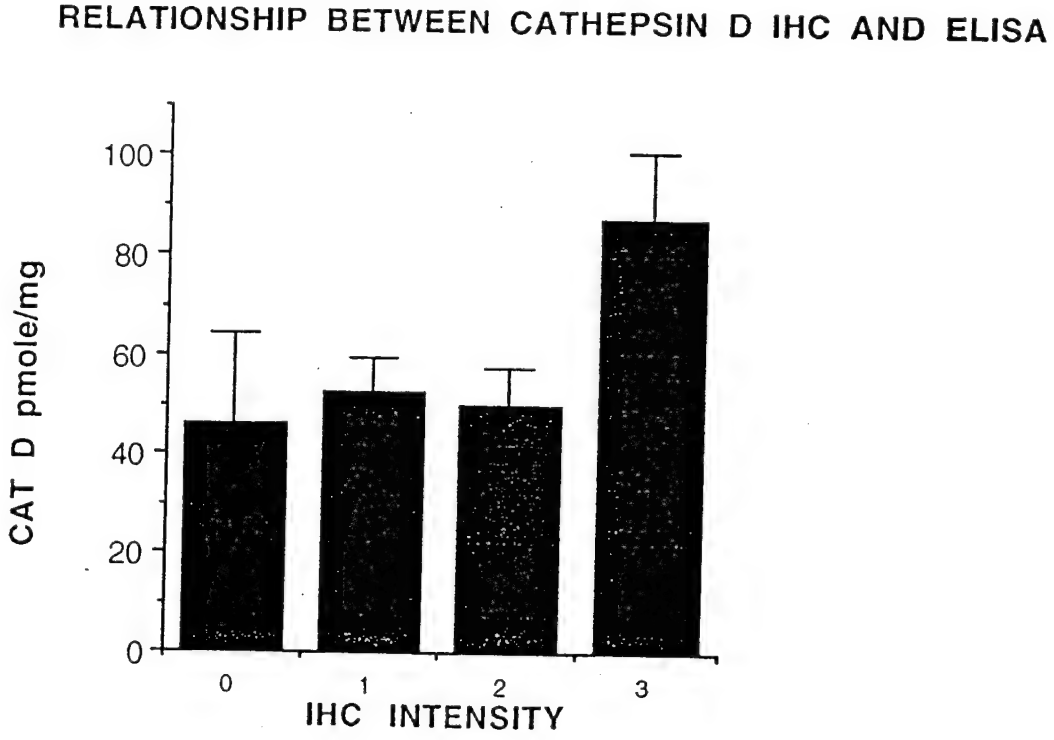
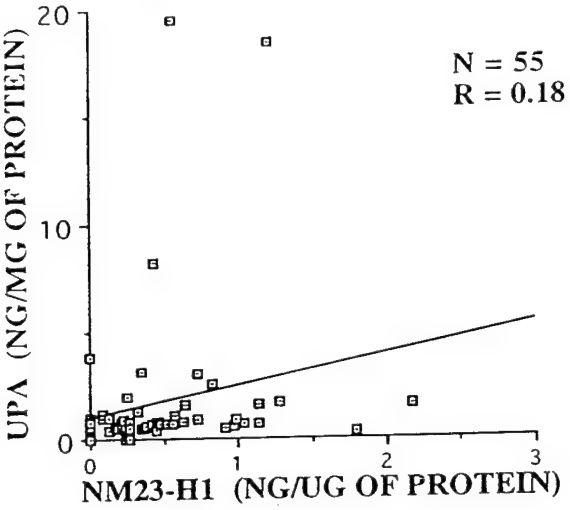
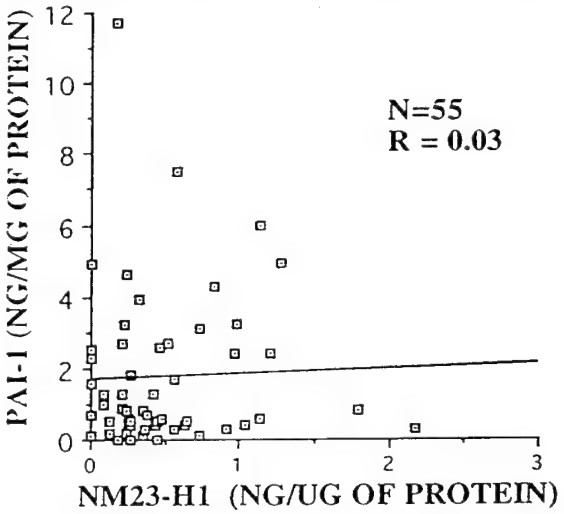


FIGURE 7

CORRELATION OF NM23-H1 WITH UROKINASE PLASMINOGEN ACTIVATOR (UPA)



CORRELATION OF NM23-H1 WITH UROKINASE PLASMINOGEN ACTAVATOR INHIBITOR (PAI-1)



CORRELATION OF NM23-H1 WITH UROKINASE PLASMINOGEN ACTIVATOR RECEPTOR (UPAR)

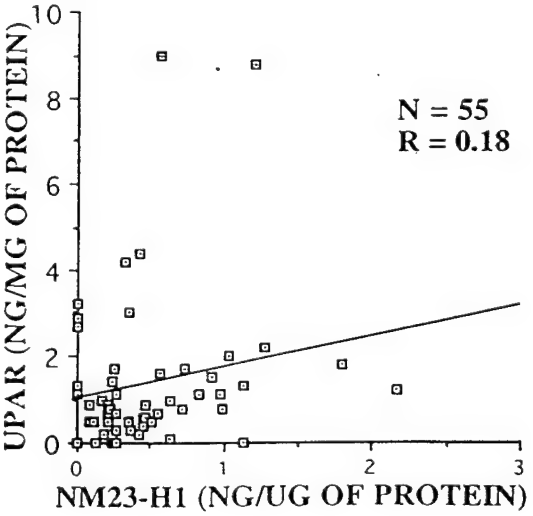


FIGURE 8

NM23 concentration in human breast cancer grown as xenografts in nude and the correlation with metastatic potential.

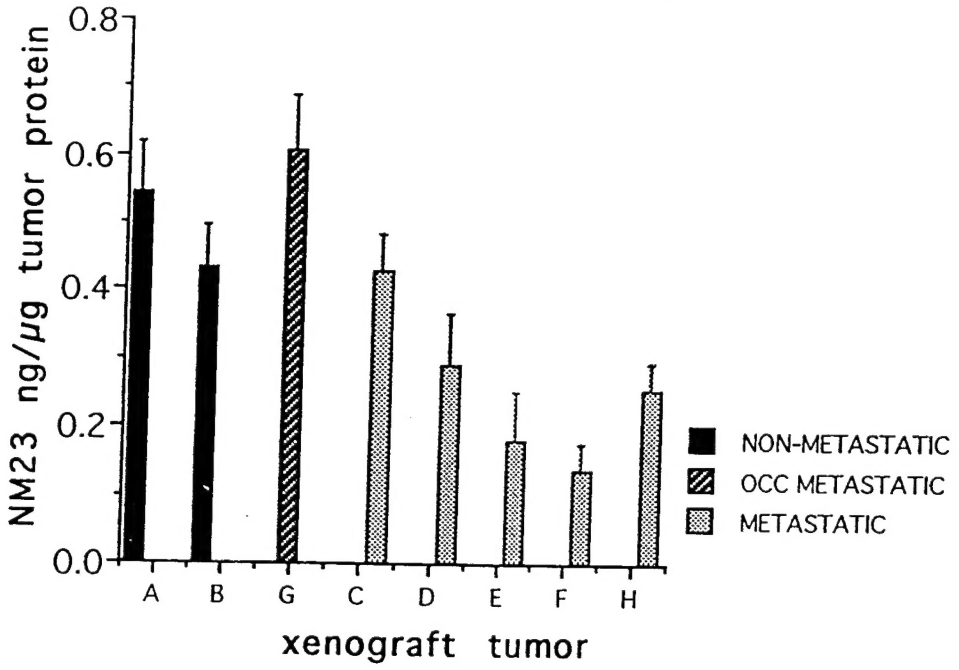


FIGURE 9

COMPARISON OF CATHEPSIN D LEVELS IN PRIMARY BREAST TUMOR AND THE RELATIONSHIP TO METASTATIC POTENTIAL

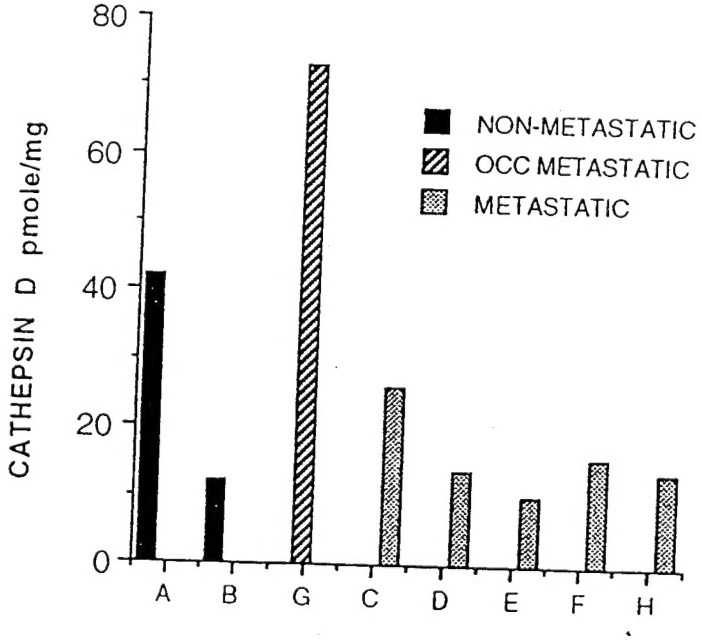
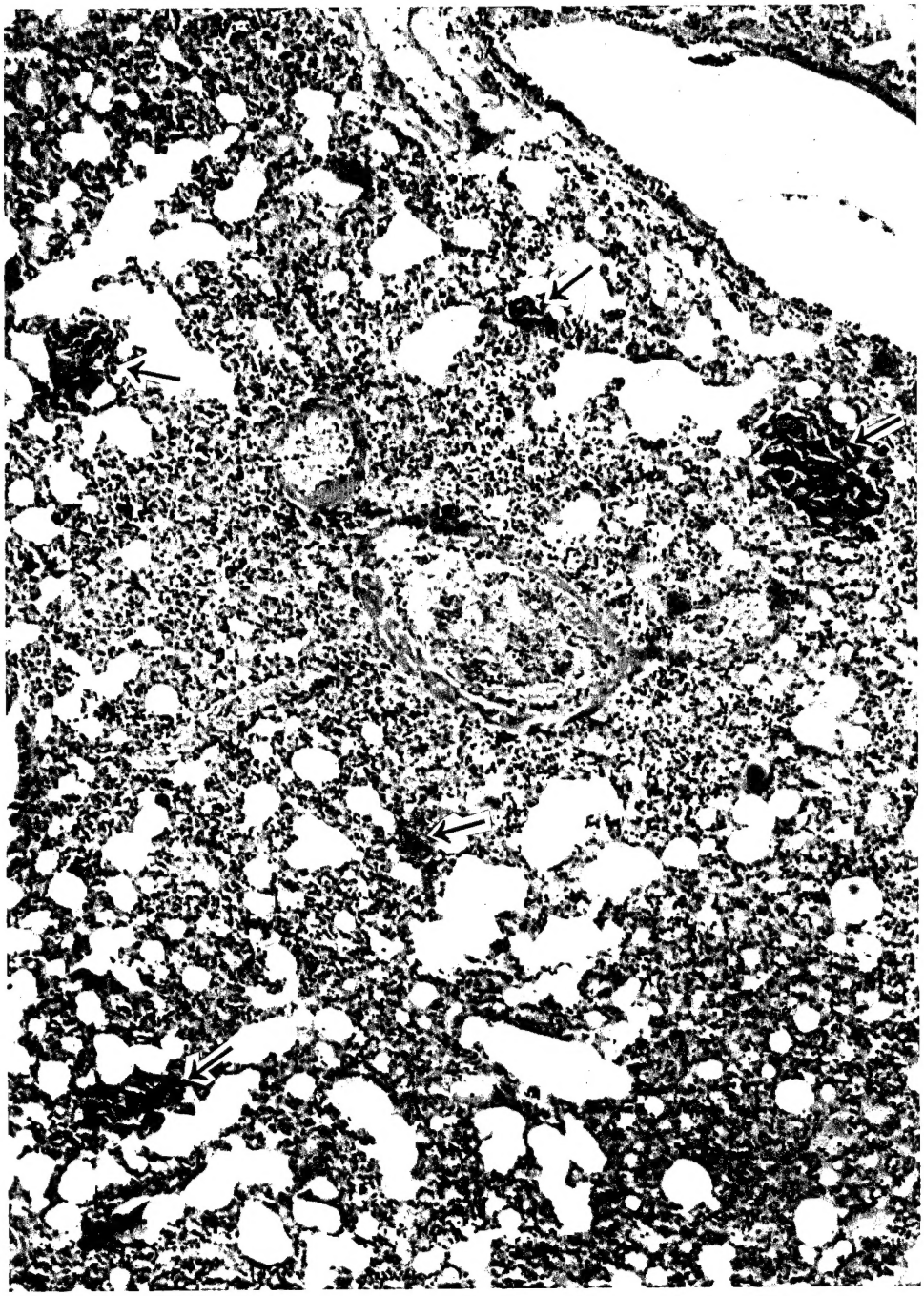


Figure 10A

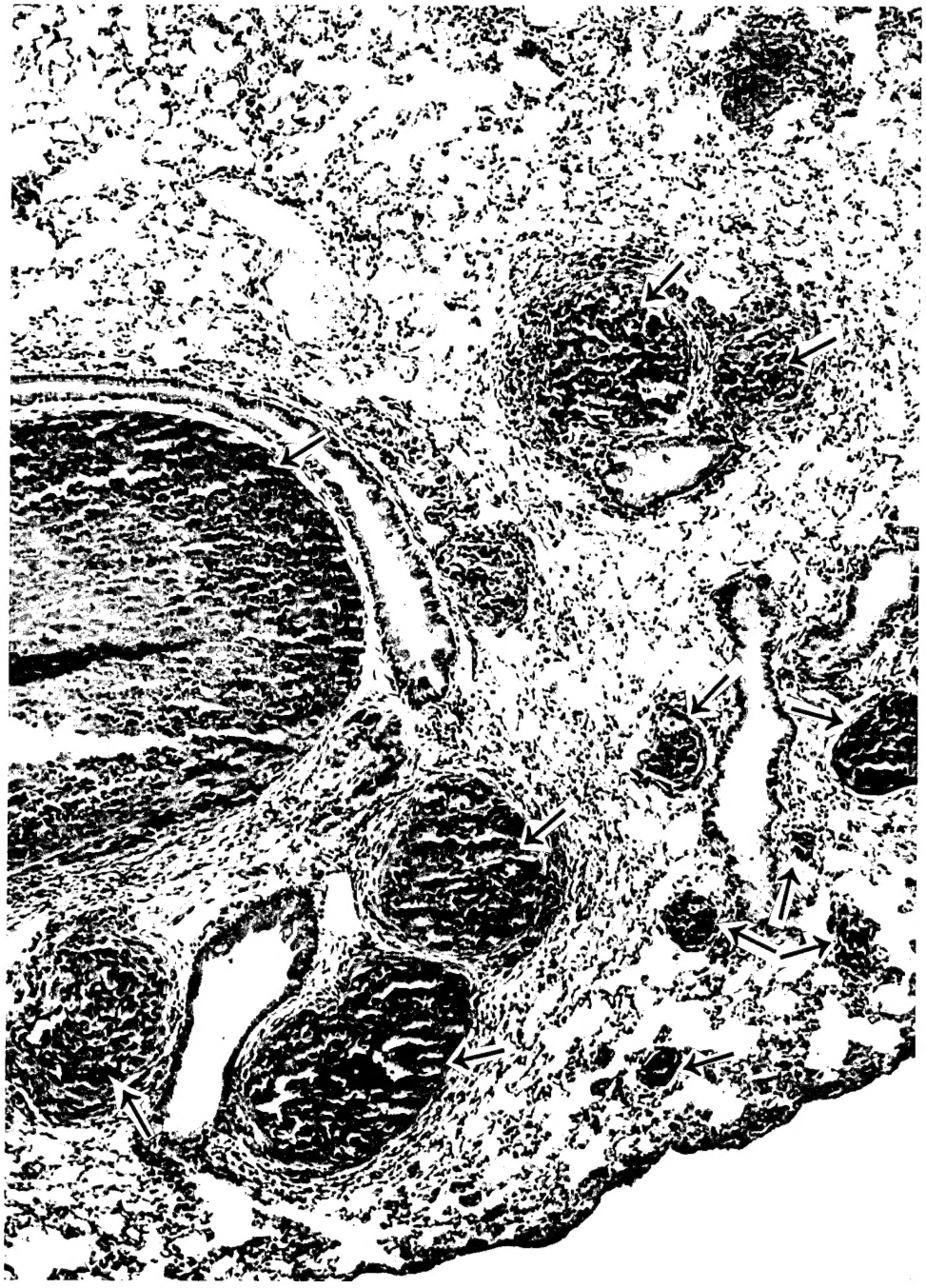
LACZ ANALYSIS FOR



MDA-MB-231 IN LUNGS

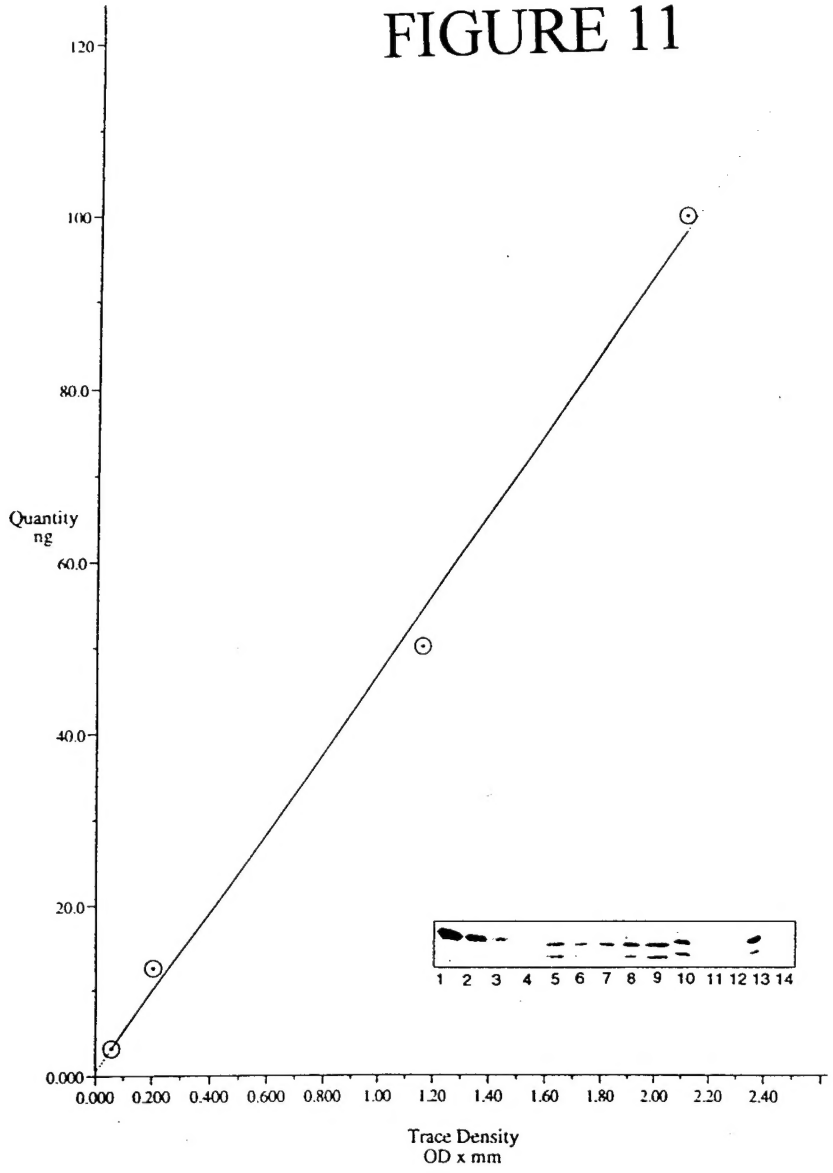
Figure 10B

LACZ ANALYSIS FOR:



MDA-MB- 435 IN LUNGS

FIGURE 11



Relationship between the fold induction of nm23 and cathepsin D in MDA-MB-231 nm23 transfected clones

FIGURE 12